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Wide range quantitative photoacoustic spectroscopy to measure non-linear optical absorption of hemoglobin

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ABSTRACT

Photoacoustic microscopy (PAM) has been shown to be a valuable tool for quantifying hemoglobin oxygenation within single vessels. Recently, optical-resolution PAM was developed to achieve higher resolution by reducing the laser beam diameter, which increased the light intensity. As intensity increases, saturation of the optical absorption and multi-photon/multi-step absorption can occur, which, together with the temperature dependence of thermal expansion, result in a non-linear dependence of the photoacoustic signal on the excitation pulse fluence. For hemoglobin, the major absorber in tissue for photoacoustic imaging, these non-linear phenomena have strong wavelength dependence. To enable quantitative photoacoustic measurements at different wavelengths in the presence of nonlinearity, a careful wide range analysis of the intensity-dependent absorption is required. Here, we built a photoacoustic spectrometer, using a tunable nanosecond optical parametric oscillator that operates between 410 nm and 2400 nm as our light source. To reduce uncertainty in our measurements due to inhomogeneous spatial distribution of the optical fluence, we used a flat-top beam illumination and a flat transducer which was mounted in reflection mode, effectively reducing quantitative measurements to a one dimensional problem. Intensity-dependent non-linear spectra of the photoacoustic signals of oxy- and deoxy-hemoglobin were obtained. These measurements have the potential to contribute significantly to quantitative functional PAM.

Keywords: Photoacoustic microscopy; non-linear photoacoustic microscopy; oxygen saturation; optical saturation; photoacoustic tomography; OR-PAM

1. INTRODUCTION

1.1 Quantitative photoacoustic microscopy

Photoacoustic microscopy is an effective functional and molecular imaging tool in vivo. Because the photoacoustic (PA) signal is proportional to the deposited optical energy, which in turn is proportional to the local optical absorption coefficient, multi-wavelength photoacoustic measurements provide quantitative information about the concentrations of multiple chromophores, such as oxygenated and deoxygenated hemoglobin molecules in red blood cells. Thus, the relative concentration and the oxygen saturation (sO_2) of hemoglobin can be extracted¹⁻³. All quantitative functional photoacoustic studies have assumed a linear dependence between the photoacoustic signal and the local optical fluence, and used the linear least squares model. Recently, we showed that non-linear effects, such as saturation of the optical absorption, thermal dependence of the Grueneisen coefficient, and/or multi-photon/multi-step excitation affect the quantitative measurements both in vitro and in vivo⁴. For hemoglobin, the major absorber in tissue for photoacoustic imaging, these non-linear phenomena have strong wavelength dependence. The objective of this paper is to demonstrate the intensity-dependent non-linear spectra of the PA signals of oxy- and deoxy-hemoglobin and to identify wavelengths most beneficial for quantitative PAM.

1.2 Non-linear photoacoustic microscopy

In an absorbing medium, the photoacoustic (PA) signal, q , generated by single photon absorption is assumed to be proportional to the local pressure rise⁵:

$$q(F) \propto \Gamma \cdot \eta_{th} \cdot \mu_a \cdot F, \quad (1)$$

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where Γ is the Grueneisen parameter (dimensionless), η_{th} is the percentage of absorbed energy that is converted to heat, μ_a is the optical absorption coefficient (cm^{-1}), and F is the local optical fluence (J/cm^2). The local optical fluence can also be written as $F = I \cdot \tau_{laser}$, where I is the local laser intensity (W/cm^2) and τ_{laser} is the laser pulse width. At low intensity, the absorption coefficient can be written as the absorption cross section, σ_a , multiplied by the number of absorbers per unit volume, N_0 :

$$\mu_a = \sigma_a \cdot N_0, \quad (2)$$

As intensity increases, mechanisms such as saturation of the optical absorption or multi-photon/multi-step absorption can occur, resulting in a non-linear dependence of the photoacoustic signal on the excitation pulse fluence⁶⁻⁸

$$q(F) \propto \Gamma \cdot \eta_{th} \cdot \sigma_a \cdot \frac{N_0}{1 + \frac{I}{I_{sat}}} \cdot F + k_1 \cdot F^2, \quad (3)$$

where I_{sat} is the saturation intensity and k_1 is the two-photon / multi-step absorption coefficient. The saturation intensity is at minimum when the absorption cross section is at maximum. For example, for oxyhemoglobin, the photoacoustic signal saturates at wavelengths near the absorption peaks at 578 nm and 540 nm (see Figure 1).

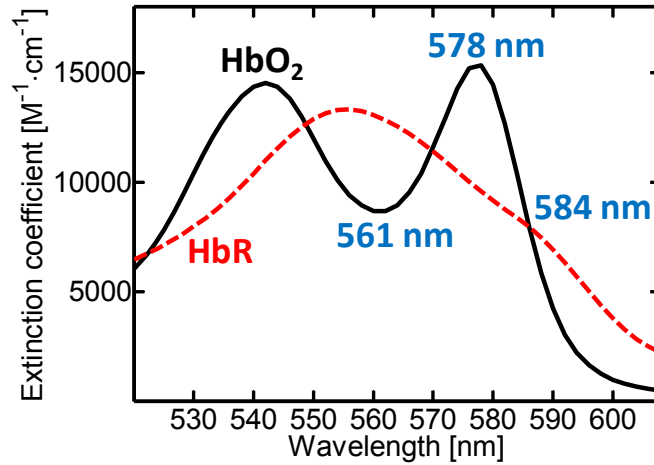


Figure 1: Molar extinction coefficient of oxyhemoglobin (black line) and deoxyhemoglobin (dashed red line).

Moreover, when the temperature rise due to the absorption is high, the Grueneisen parameter, which depends on the temperature, changes⁹:

$$\Gamma(T) \cong \Gamma(T_0) + \frac{\partial \Gamma}{\partial T} \cdot \Delta T = \Gamma_0 + k_2 \cdot \mu_a \cdot F, \quad (4)$$

$\Delta T \propto \mu_a \cdot F \quad \uparrow$

where k_2 is a linear approximation coefficient of the Grueneisen parameter. The resulted photoacoustic signal is

$$q(F) \propto (\Gamma_0 + k_2 \cdot \mu_a \cdot F) \cdot \eta_{th} \cdot \mu_a \cdot F = \Gamma_0 \cdot \eta_{th} \cdot \mu_a \cdot F + k_2 \cdot \eta_{th} \cdot (\mu_a \cdot F)^2. \quad (5)$$

2. EXPERIMENTAL SETUP AND RESULTS

2.1 Flat-top PAM

We measured the photoacoustic signal as a function of the incident laser fluence, using a custom-built PAM system with a flat top beam illumination shown in Figure 2. A Q-Switched Nd:YAG laser (Quatel Inc.) with 3rd harmonic (355 nm) output was used to pump an OPO laser (BasiScan, Newport), which provided lasing at 10 Hz in the wavelength range of 410-1050 nm. The ultrasonic and optic coupling cube was made of a BK7 glass prism joined with rhomboidal prism with a layer of index matching liquid between them. The liquid layer allows light beam to pass freely towards a sample cell, while reflecting the ultrasound pulse coming from the sample. Ultrasonic reflection occurs with near 100%

conversion into a shear wave, which after second reflection from a free surface, is converted back to longitudinal wave and detected by an attached 30 MHz ultrasonic transducer (V213-BC, Olympus). A 3 mm thick Teflon sample cell with 3 mm inner diameter was attached to the prism. Several micro-liters of dye solution of interest were deposited into the cell on the prism surface. During the experiments, the cell was fully filled with solution to decrease the effect of photobleaching and to assure constant concentration of the dye. However, equivalent thickness of the cell is roughly equal to the ultrasonic wavelength in solvent, which is approximately 50 μm . Hence, minimal amount of solution can be as small as 50 nL. To measure the laser pulse energy, a small portion of the light was reflected to a pyroelectric detector (SPH-11, Spectrum Detector Inc.), which was calibrated for a wide range of laser pulse energies over the entire wavelength range. The signals from the ultrasonic transducer and the pyroelectric detector were digitized using a 14 bit, 200 MHz digitizer (14200, Gage). To minimize non-linear dependence of the photoacoustic signal on the beam profile, the laser beam profile was kept equally constant across the area of illumination using an engineered diffuser (PRC Photonics) that was incorporated into the cell design. This diffuser provided 5-10% uniformity within a 1 mm optical beam diameter at the sample cell surface (see Figure 2). The diffuser had a high laser damage threshold, and a broad transmission spectrum spanning from 180-2500 nm.

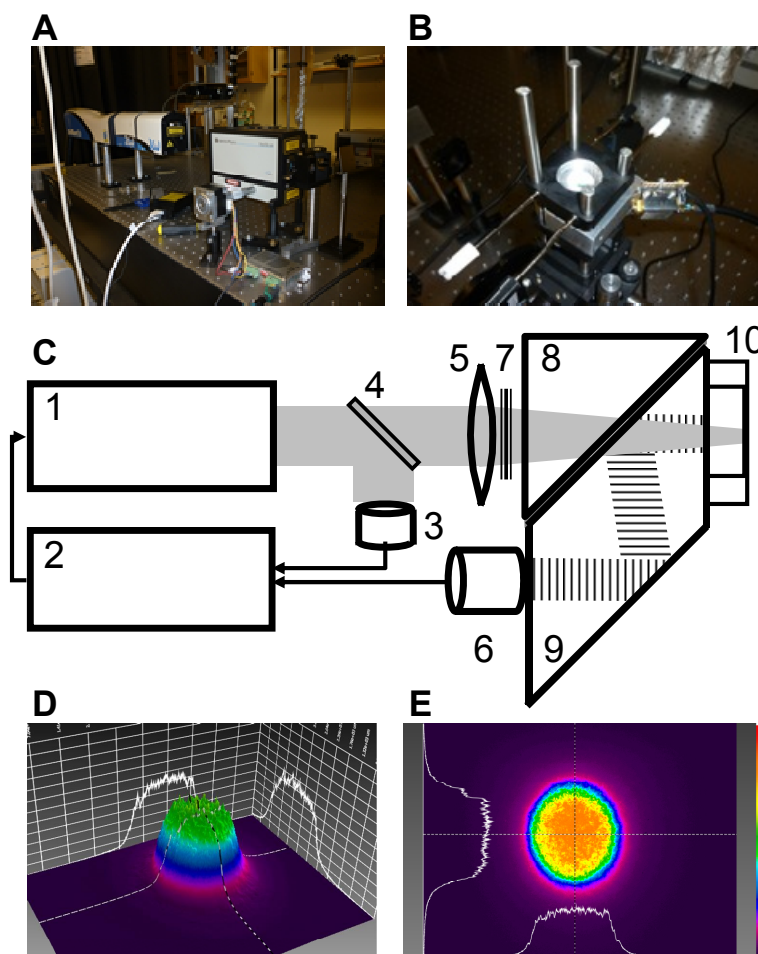


Figure 2: PAM system with a flat top beam illumination. A. Photoacoustic spectrophotometer, B. photoacoustic cell, C. Schematic of the photoacoustic spectrophotometer: 1. Tunable pulse laser, 2. Data acquisition system, 3. Pyroelectric sensor, 4. Beam sampler, 5. Focusing lens, 6. Ultrasonic transducer, 7. Engineered diffuser, 8. Right angle prism, 9. Rhomboidal prism, 10. Dye cell, D. Beam profile (3D), E. Beam profile (2D).

2.2 Oxygenated hemoglobin

We measured the photoacoustic signal (maximum peak to peak amplitude) from HbO_2 and HbR as a function of the incident pulse energy in the wavelength range 450-650 nm. The PA signal at each wavelength was averaged using 20

pulses and normalized by the averaged pulse energy. The pulse repetition rate was 10 Hz. Oxygenated lysed bovine blood (50:50 DI-water and defibrinated bovine blood) was purchased from Quadfive (#905-250) and further diluted (1:10) with DI-water. The oxygenated level was measured to be 100% using a spectrophotometer.

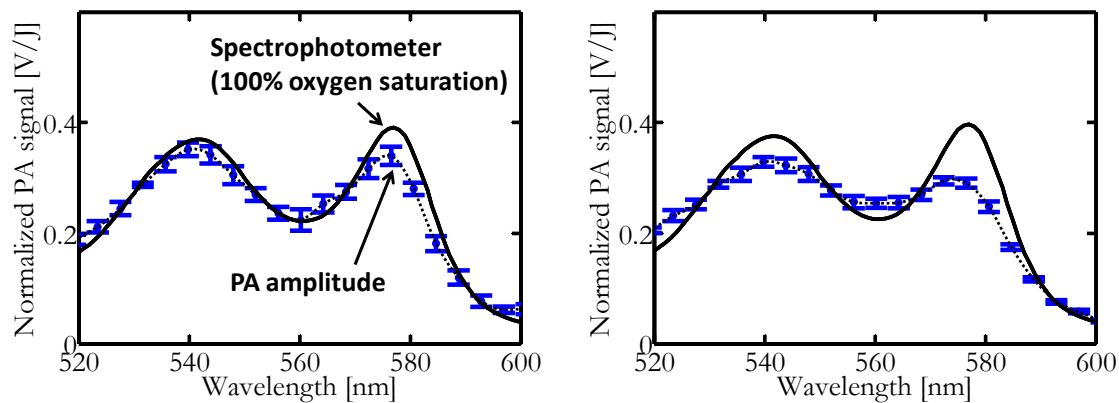


Figure 3: The PA amplitude as a function of the wavelength in fully oxygenated hemoglobin at (a) Low fluence, and (b) high fluence. The solid line is the measured absorption using a spectrophotometer and it fits the theoretical absorption of 100% oxygenated hemoglobin.

The PA amplitude as a function of the incident fluence is presented in the following figures for different wavelengths.

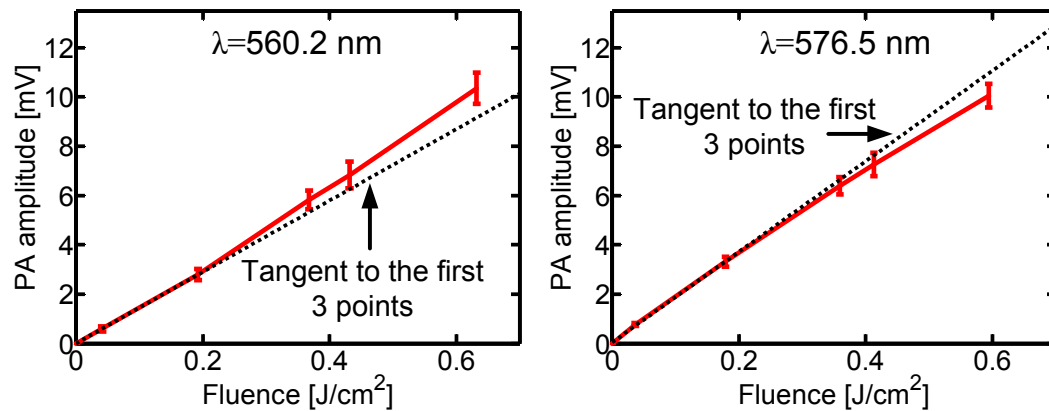


Figure 4: The PA amplitude as a function of the incident fluence at different wavelengths.

At the absorption peaks (576 nm and 540 nm) of oxy-hemoglobin, saturation is the dominant non-linear phenomenon. At other wavelengths (e.g., 560 nm), the dominant non-linear phenomenon has quadratic dependent, suggesting either thermal non-linearity or multi-step excitation.

2.3 Deoxygenated hemoglobin

Deoxygenated blood was measured in a dead mouse tissue. The mouse was euthanized by putting it into a chamber of 100% carbon dioxide, which induced hypoxemia. After confirming death, the mouse was put in a fridge for one hour. The scalp and skull were then removed, and a thin slice of brain tissue was excised. The tissue was mainly gray matter, which is rich in capillary beds.

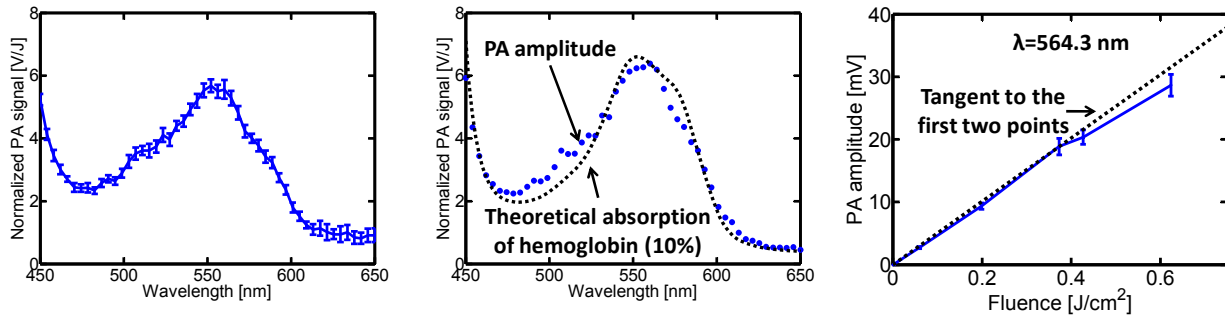


Figure 5: (a) The PA amplitude as a function of the wavelength in deoxygenated hemoglobin (b) The fitted theoretical absorption of 10% oxygen saturated hemoglobin (c) The PA amplitude as a function of the incident fluence at 564.3 nm

2.4 Wavelength dependent quadratic coefficient

To evaluate the amount and type of non-linearity, at each wavelength we fitted the photoacoustic amplitude as a function of the fluence to a second order polynomial function:

$$PA = a_1 \cdot F + a_2 \cdot F^2 \quad (6)$$

When the quadratic coefficient is negative, the dominant non-linear effect at the corresponding wavelength is saturation. When the quadratic coefficient is positive, the dominant non-linear effect is thermal non-linearity or multi-step excitation.

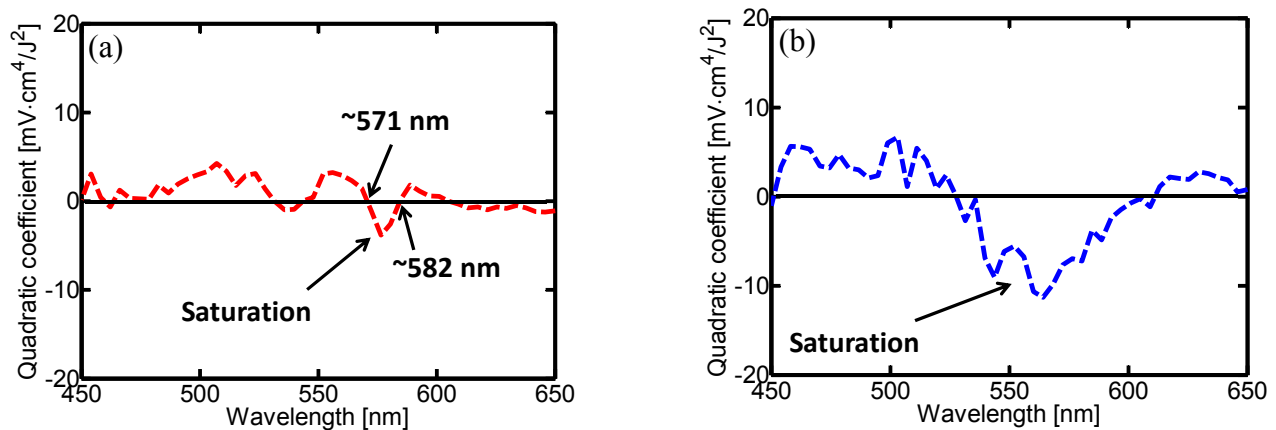


Figure 6: The quadratic coefficient as a function of the wavelength in (a) oxy-hemoglobin and (b) deoxy-hemoglobin.

3. CONCLUSIONS

We showed that at high intensity the PA signal generated from oxy- and deoxy-hemoglobin exhibits non-linear effects that are wavelength dependent. The dominant non-linear effect near the absorption peaks is saturation. However, away from the peaks, either thermal non-linearity or multi-step excitation dominates. In particular, some wavelengths were found to have less non-linearity than other wavelengths even at high intensity. Measurements made at these wavelengths are preferable for quantitative functional PAM, where linearity is a fundamental assumption.

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